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By: Karen Karlin

PATENT
Attorney Docket No.: 015280-358100US
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: AMBS and HARRIS Application No.: 09/830,977 Filed: July 31, 2001 For: P53 AND VEGF REGULATE TUMOR GROWTH OF NOS2 EXPRESSING CANCER CELLS Customer No.: 20350	Confirmation No. 7226 Examiner: Sheela Jitendra Huff Technology Center/Art Unit: 1642 <u>Declaration of Stefan Ambs and Curtis C. Harris pursuant to 37 C. F. R. §1.131</u>

Commissioner for Patents
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Sir:

We, Stefan Ambs and Curtis C. Harris, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both, under 18 U.S.C. §1001, and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of our own knowledge are true and statements made on information or belief are believed to be true. Exhibit I is attached hereto and is incorporated herein by reference.

2. At the time this invention was first conceived, we were employees of the National Cancer Institute, located in Bethesda, Maryland. All activities described in this Declaration took place in the United States of America.

3. In accordance with 37 C.F.R. §1.131, we state that we completed the claimed invention in the United States prior to November 5, 1998, which is the publication date under 35 U.S.C. §102(a) for the Reiger *et al.* reference (*Oncogene* 1998, **17**:2323-2332).

4. Attached to this Declaration are Exhibit I, a manuscript describing this invention, and Exhibit II, an Employee Invention Report containing our signatures. The dates in the Exhibits have been redacted. All redacted dates are prior to November 5, 1998.

5. Conception of the present invention as well as its reduction to practice are evidenced by Exhibits I and II. On page 18 of Exhibit I, it is described that a "NOS2 inhibitor, aminoguanidine (1%AG), suppressed the tumour growth of NOS2-expressing Calu-6 cells," which lack functional p53. The experimental results demonstrating this growth suppression are shown in Figure 1c (D) of Exhibit I. Exhibit II further demonstrates that the use of a cell-based assay for screening of candidate therapeutic agent had been conceived (see paragraph 8 on page 2 of Exhibit II).

6. In light of the foregoing, it is established that Declarants had in their possession the claimed subject matter of the present invention prior to November 5, 1998.

7. Declarants have nothing further to say.

Dated: 7/7/04 By: ______________________
Stefan Ambs, Ph.D.

Dated: 7/7/04 By: ______________________
Curtis C. Harris, M.D.

Attachments (Exhibits I and II: redacted copies of manuscript and Employee Invention Report)
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Nature Medicine

p53 and vascular endothelial growth factor regulate tumour growth of NOS2-expressing human carcinoma cells

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The finding of frequent nitric oxide synthase (NOS) expression in human cancers suggests a pathophysiological role of nitric oxide (NO) in carcinogenesis. To define a role of NO in tumour progression, we generated human carcinoma cell lines that produced NO constitutively. The NOS2-expressing cancer cells with wild-type p53 had reduced tumour growth in athymic nude mice, whereas the NOS2-expressing cancer cells with mutated p53 had accelerated tumour growth associated with increased vascular endothelial growth factor expression and neovascularization. Our data indicate that tumour-associated NO production may promote cancer progression by providing a selective growth advantage to tumour cells bearing mutant p53, and that NOS2 inhibitors may have therapeutic activity in these tumours.

Increased expression of inducible nitric oxide synthase (NOS2) has been found in a variety of human cancers [1-4], and a NOS2-specific inhibitor can reduce growth of xenografted tumours in mice [5]. Hypoxia upregulates NOS2 expression [6], and NO induces mitogenesis among endothelial cells [7]. Recently, NO has been shown to induce vascular endothelial growth factor (VEGF) expression in carcinoma cells [8], and tumour neovascularization [4,9]. Thus, the promotion of tumour growth by NO [9,10] may involve the induction of angiogenic factors [11]. However, the function of NO in carcinogenesis is uncertain: NO has been found to either inhibit [12] or stimulate tumour growth [9,10]. High concentrations of NO are also known to induce cell death in many cell types including tumours cells [13-15], whereas lower concentrations of NO can have an opposite effect and protect against apoptotic cell death from various stimuli [16,17]. We investigated the role of NO in tumour growth using carcinoma cells genetically engineered to produce NO constitutively, and found that the effect of NO on tumour

growth is p53-dependent, and that endogenously produced NO accerelerates tumour growth by inducing VEGF and neovascularization.

Constitutive expression of NOS2 in human carcinoma cell lines

High concentrations of NO induce p53 accumulation and p53-mediated growth arrest or apoptosis [18,19]. To investigate the functional interaction of p53 and NO in tumour growth, we infected human carcinoma cells, which had a wild-type, missense mutant or p53 null status, with a retroviral construct, DFG-iNOS [20]. The amounts of NO produced by 10^6 of these cells ranged from 2 to 15 nmole of nitrite plus nitrate per day (Table 1), which is significantly lower than NO production in cytokine-stimulated macrophages [21]. Isogenic vector-control carcinoma cell lines, that expressed β-galactosidase (β-gal) instead of human NOS2 [20], did not produce detectable amounts of NO.

NOS2 expresssion and tumour growth

We investigated the effects of NOS2 expression on the growth rates of human carcinoma cells both in cell culture and in subcutaneous tumours in athymic nude mice. In cell culture, NOS2-expressing carcinoma cell clones grew at the same rate as the isogenic vector controls (Fig. 1a). Though NO cytotoxicity has been described in tumour cells after transfection with murine NOS2 [9,13], it was not observed in the DFG-iNOS infected cell clones, which is consistent with the moderate NO production in these cell lines. To further evaluate whether NO alters tumour growth, NOS2- or β-gal-expressing carcinoma cells were subcutaneously inoculated into athymic nude mice and tumour growth was monitored. NO-producing LoVo cells that expressed wild-type p53 grew slower and produced smaller

tumours than the isogenic vector controls (Fig. 1b). In contrast, NO-producing Calu-6 cells that are p53 null grew faster and produced larger tumours than the isogenic vector control cells (Fig. 1b). The observation that NO affects tumour growth depending on the p53 status was extended by additional studies. NO affected tumour growth in a dose-dependent manner (Fig. 1d), and also reduced the tumour growth of both colon carcinoma cell lines with wild-type p53, RKO and HCT-116 cells, while it accelerated the growth of a colon carcinoma cell line homozygous for mutant p53 (codon 273^{His}), HT-29 cells (Fig. 1c). The tumours derived from NOS2-expressing LoVo, Calu-6 and RKO cells contained NOS2 activities comparable to those frequently found in a cohort of colorectal tumours [3] and ranged from 3 to 25 pmole/min/mg. Furthermore, aminoguanidine, a specific inhibitor of NOS2 [22], significantly reduced the tumour growth of NOS2-expressing Calu-6 ($p<0.05$, two-tailed Student's t-test, Fig. 1b) and HT-29 cells ($p=0.002$, Kaplan-Meier analysis, Fig. 1c).

NO-induced neovascularization

We next investigated mechanisms whereby endogenous NO production could accelerate the tumour growth of carcinoma cells which are either null or mutant for p53. NO has angiogenic properties and has been shown to increase the number of blood vessels in tumours grown by DLD-1 human colon carcinoma cells transfected with murine NOS2 [9]. Therefore, we analyzed subcutaneous tumours produced by Calu-6 cells in nude mice for angiogenesis by performing immunohistochemistry for CD31, which is a specific marker of endothelial cells and vascularization [23]. We found that tumours expressing NOS2 contained significantly ($p<0.01$, two-tailed Student's t-test) more small blood vessels than tumours lacking NOS2 (Fig. 2a). Vector control tumours contained large necrotic areas

not found in tumours expressing NOS2, and we speculate that deficient angiogenesis limited the growth of these controls. Our observations are consistent with reports linking endogenous NO production to an increased tumour growth rate, presumably by enhancing angiogenesis [9]. Based on these observations, the lack of an aminoguanidine effect in slow-growing tumours of NOS2-expressing LoVo cells might be explained by insufficient microvascularization, i.e., not allowing an effective inhibitor concentration, while the more vascular tumours of NOS2 expressing Calu-6 cells were inhibited by higher concentrations of aminoguanidine (Fig. 1b).

Increased vascular endothelial growth factor expression in NOS2 expressing cells

To explore the angiogenic activity of NO, we investigated VEGF as a downstream effector. NO is capable of depleting the intracellular iron storage by which it activates the IRE binding protein [24]. Iron depletion also activates VEGF expression [25]. Therefore, we investigated VEGF mRNA and protein expression in carcinoma cells expressing NOS2. VEGF protein concentrations were higher in cellular extracts of NO expressing clones than in extracts of the vector control cell lines (Fig. 2b). To further buttress this finding, we determined VEGF mRNA levels in Calu-6 cells. VEGF mRNA steady state concentrations were increased in two NOS2-expressing cell clones when compared to the β-gal-expressing vector control (Fig. 2c). The VEGF mRNA expression levels also correlated with an increased secretion of VEGF protein into the culture medium (Fig. 2c). The addition of a NOS inhibitor, N^G-monomethyl-L-arginine (L-NMA), to the cell culture medium reduced the VEGF secretion. These results demonstrate that endogenously produced NO increases VEGF secretion in human carcinoma cells which is consistent with a recent report showing that NO-donors induce

guanylate cyclase-dependent upregulation of VEGF mRNA [8]. Additionally, an increased VEGF mRNA level was found in tumours of NOS2-expressing Calu-6 cells (data not shown).

Discussion

We report here that endogenously produced NO inhibits growth of tumour cells with wild-type, but not mutant, p53 in the athymic nude mouse model, and induces VEGF expression and angiogenesis. A reciprocal relation between VEGF and NO has recently been observed with an angioplasty model in which NO inhibits protein kinase C-dependent VEGF expression in smooth muscle cells [26]. We found an opposite relationship in human tumour cells, and confirm recent data showing that NO released by a NO-donor upregulates VEGF mRNA levels in human carcinoma cells [8]. The increase of VEGF mRNA involves the guanylate cyclase pathway [8], and may arise from an increased stability of the VEGF mRNA [27]. In our experiments, the upregulation of VEGF by NO coincided with increased vascularization in xenografted tumours of NOS2-expressing cells. This correlation between NOS2 expression and tumour vascularization has also been observed with xenografts of murine NOS2-transfected DLD-1 human carcinoma cells [9]. Because NOS2 expression in tumour infiltrating monocytes corresponds with the onset of VEGF expression in human colon adenomas [3], and NOS2 is induced by hypoxia [6], we speculate that induction of NOS2 is part of the early response in tumour angiogenesis allowing tumours to grow more than 1 mm^3 [28]. The observation that the NOS2-specific inhibitor 1400W limits tumour growth in mice [5] also supports this view.

NO is an activator of the p53 tumour suppressor gene function [18,19,29], and high concentrations of NO induce apoptosis in tumour cells *in vitro* [13,14]. We found that low to moderate concentrations of

endogenous NO did not inhibit the growth of human carcinoma cell lines *in vitro*. Although NO produces multiple effects on tumour growth *in vivo*, ranging from inhibition to stimulation [4,9,10,12], our data indicate that such variations may be linked not only to differences in the NO concentration but also to the functional status of the p53 tumour suppressor gene. NO triggers p53 accumulation [18,19]. However, we did not observe p53 accumulation in tumours of NOS2-expressing cells with immunohistochemical staining. The moderate NO production in our cell lines may not be sufficient to induce detectable nuclear p53, and may have activated p53 by inducing a confirmational change [29]. Whereas NO accelerated tumour growth of p53 null or mutant p53 cells *in vivo*, wild-type p53 inhibited tumour growth. Wild-type p53 is a known inhibitor of tumour angiogenesis [30]. Thus, the loss of p53 function in p53 null or mutant cells would permit both the growth of tumour cells in the presence of moderate NO concentrations and the release of angiogenic factors such as VEGF.

Increased NOS2 levels have been detected in human breast, brain, head and neck and colon cancers [1-4]. Constituitive expression of NOS2 in those tumours may lead to a p53-mediated growth arrest in the epithelial cells close to the source of NO production. As our data indicate, the resulting growth inhibition would provide a strong selection pressure for mutant p53. Indeed, breast, brain, head and neck and colon cancers that can overexpress NOS2 [1-4] have a high frequency of p53 mutations [31]. Clonal selection and growth are further supported by NO-induced VEGF expression and angiogenesis. In addition, wild-type p53 transrepresses both basal and cytokine-induced NOS2 in a negative feedback loop [19], so that NOS2 expression would be unchecked in cells with mutant p53. These data are consistent with the hypothesis that NO can act as an endogenous carcinogen in human carcinogenesis [32].

Methods

Retroviral gene transfer of human NOS2. Human carcinoma cells were infected, as described [20], with either the retroviral vector DFG-iNOS, carrying the human NOS2 gene, or with a control vector, BaglacZ, in which NOS2 is replaced with the ß-galactosidase gene. Cell clones that constitutively produced nitric oxide were isolated after 14 days of G418 selection (250-350 μg G418/ml). NOS2 and ß-galactosidase expressing HCT-116, HT-29, LoVo, RKO colon carcinoma cells, and Calu-6 lung carcinoma cells (all ATCC, Rockville, MD), were cultured in A50 medium (Biofluids, Rockville, MD) supplemented with 10% FBS, 1 mM N^G-monomethyl-L-arginine, 5 mM glutamine and 200 μg G418/ml.

Growth rates were determined by plating cells in triplicate dishes at 10^3 cells/60 mm dish and staining three dishes per day. Cells were rinsed in phosphate-buffered saline, fixed in 2% formaldehyde and stained with 0.25% crystal violet. The number of cells per colony was determined by counting the stained cells under the microscope. The number of cells was determined in 10 colonies/dish, and population doublings are expressed as log2(cells/colony).

Determination of nitrite plus nitrate. 3×10^6 cells were plated in 9 mm^2 culture wells (Costar, Cambridge, MA) and cultured in 4 ml of medium for 48 hr. To determine nitrite plus nitrate concentrations in culture medium, nitrate was converted to nitrite, and nitrite was determined with the Griess reagent [19].

Tumour xenoplantation. Suspensions of 3×10^5 to 5×10^6 cells in a volume of 0.2 ml were injected at a single subcutaneous site into athymic nude mice previously irradiated with 350 rads. Either 10 or 20 animals were injected per experiment. A nodule was scored as a tumour when it measured 125 mm^3 or more by its largest two dimensions.

CD31 immunohistochemistry. Five micron sections of ethanol-fixed tumours were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked by treatment with H_2O_2. Sections were incubated with a 1:50 dilution of normal goat serum in PBS/2% BSA and then with the MEC13.3 rat monoclonal anti-mouse CD31 antibody (PharMingen), 1:200 diluted, in PBS/2% BSA for 45 min. Slides were rinsed with PBS and incubated with a secondary, biotin-labeled anti-rat Ig antibody (Vectastain). After incubation with an avidin-biotin-peroxidase complex, slides were stained with 3,3-diaminobenzidine for 10-20 min. The counting of microvessels was performed at x250 magnification (x25 objective and x10 ocular). At this magnification, 8 areas per tumour, not including tumour edges, were scanned and all CD31-positive vessels were counted.

NOS2 and VEGF Western blot analysis. Cell lysates for Western blotting were prepared by solubilization of cell pellets in RIPA buffer [3]. VEGF protein concentrations were determined as follows. Five µg of rabbit polyclonal anti-VEGF antibody (Santa Cruz Biotechnology) were added to either 1 mg of cellular protein extract or 1 ml of cell culture medium, incubated for 1 hr at 8-10°C, and then mixed with protein A-sepharose (10 mg) for 1 hr. Samples were spun at 10,000 g, and pellets were washed with RIPA buffer, boiled in SDS/DTT buffer (5,3-Prime) and loaded on a SDS/13%

polyacrylamide gel. For NOS2, 100 μg of soluble protein extract were loaded on a SDS/7% polyacrylamide gel. After transfer to an Immobilon-P membrane (Millipore), NOS2 and VEGF protein were detected with either a polyclonal anti-NOS2 antibody (Merck), 1:40,000 diluted, or a polyclonal anti-VEGF, 1:1000 diluted, as described [3].

Northern blotting. Total cellular RNA was prepared with the RNeasy™ kit (QIAGEN). 30-50 μg of RNA were resolved on a 1.2% agarose gel containing 6.3% formaldehyde, transferred to a Hybond™-N nylon membrane (Amersham) and hybridized with a ^{32}P-labeled cDNA probe containing either the full-length human NOS2 sequence [33] or 522 bp of the human VEGF sequence common for all known VEGF isoforms. The VEGF cDNA was generated by RT-PCR (Advantage™RT-for-PCR kit, Clontech) using RNA from HCT-116 human colon carcinoma cells. PCR: 32 cycles, 1 min at 58°C, at 72°C and at 94°C using Taq polymerase (Perkin Elmer); cDNA primers: 5'-GCCTCCGAAACCATGAACTTTC-3', 5'-CGAGTCTGTGTTTTTGCAGGAAC-3'.

Statistical Analysis. The Kaplan-Meier survival analysis was used to calculate the statistical significance of tumour probabilities in different treatment groups. Other comparisons were carried out by the two-tailed Student's t-test. Relationships are considered statistically significant when $p<0.05$.

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Table 1. Nitric oxide production* in human carcinoma cell lines that constitutively express NOS2

Cell line			Nitrite plus nitrate nmole/day/1x10^6 cells
Calu-6	BaglacZ		ND
	NOS2	Clone 5	8
	NOS2	Clone 7	11
LoVo	BaglacZ		ND
	NOS2	Clone 9	6
RKO	BaglacZ		ND
	NOS2	Clone 5	6
HCT-116	BaglacZ		ND
	NOS2	Clone 1	2
	NOS2	Clone 2	3
	NOS2	Clone 3	4
HT-29	BaglacZ		ND
	NOS2	Clone 1	3
		Clone 2	8
		Clone 3	15

* Determined as nitrite plus nitrate accumulation in the cell culture medium

ND - not detectable

Figure legends

Fig. 1 NOS2 expression and tumour growth. **a**, NO production in human carcinoma cells does not change cell growth in cell culture. NOS2 expressing Calu-6 and LoVo carcinoma cells were cultured both with and without 2 mM of the NOS inhibitor, N^G-monomethyl-L-arginine (L-NMA). Clonal cell growth was compared to vector controls (BaglacZ). Each point represents the average clonal growth of 10 colonies per dish in three dishes. **b**, Tumour probability of NOS2-expressing human carcinoma cell lines is dependent on the p53 status. $3x10^6$ cells of NOS2-expressing Calu-6 and LoVo carcinoma cells, and the vector controls (BaglacZ), were inoculated into 10 athymic nude mice, respectively. NOS2-expressing LoVo cells, which have two wild-type p53 alleles, grow slower (A) and produce smaller tumours (C) than vector controls (BaglacZ). In contrast, NOS2-expressing Calu-6 cells, which lack expression of functional p53, grow faster (B) and produce larger tumours (D) than the vector controls. The NOS2 inhibitor, aminoguanidine (1% AG), suppressed the tumour growth of NOS2-expressing Calu-6 cells (D, * $p<0.05$, two-tailed Student's t-test) but not vector controls. **c**, The NOS2 inhibitor aminoguanidine reverses the growth stimulatory effect of NOS2 in tumours of HT-29 colon carcinoma cells. $3x10^5$ cells of NOS2 (Retro-HNOS) and ß-galactosidase (BaglacZ) expressing HT-29 cells were inoculated into 40 athymic nude mice, respectively. Half of the animals in both groups received 1% AG in the drinking water. The tumour probability of HT-29 cells is significantly increased by NOS2 when compared to the vector controls (Kaplan-Meier survival analysis: $p=0.002$). This effect is abolished ($p=0.002$) through treatment with 1% AG. **d**, Tumour probability of NOS2 expressing colon carcinoma cell lines correlates with NO production and the p53 status. $5x10^5$ cells of

NOS2 expressing HT-29 cell clones and 1x10^6 cells of NOS2 expressing HCT-116 cell clones were inoculated into 10 athymic nude mice, respectively. The tumour probability of HT-29 cells, which carry a mutant p53, correlates positively with NOS2 activity (A) while the tumour probability of HCT-116 cells, which have wild-type p53, shows an inverse correlation with NOS2 activity (B). The relative NOS2 activity in HT-29 cells (A), measured as nitrite plus nitrate production in cell culture, is 1x for clone 1 (●), 2.7x for clone 2 (■) and 5x for clone 3 (▲). In HCT–116 cells, the relative activities are 1x in clone 1 (●), 1.5x in clone 2 (■) and 2x in clone 3 (▲).

Fig. 2 NO induces tumour micro-vascularization and VEGF expression. **a**, Immunohistochemical analysis of the endothelial cell antigen, CD31, in tumours grown by NOS2 (A) or β-galactosidase (B) expressing Calu-6 lung carcinoma cells in athymic nude mice. Numerous capillaries are stained in tumours grown by NOS2-expressing Calu-6 cells (A, arrows). In panel B, scanning magnification shows staining of only one longitudinal section of a large blood vessel (LBV) in tumours grown by the vector control cells; several necrotic areas (NA) are nearby. Number of CD31-positive microvessels per x250 field: 6.1±2.8 (NOS2) versus 0.7±0.7 (vector control); $p<0.01$, two-tailed Student's *t*-test. Methyl green counterstain. Magnification: A and B, x100. **b**, Increased VEGF concentration in protein extracts of NOS2-expressing human carcinoma cell lines. Protein extracts were prepared from RKO, HCT-116, HT-29, Calu-6 and LoVo cells infected with the retroviral construct DFG-iNOS. The NOS2 protein band at 130 kDa was detected by Western blot analysis with a polyclonal anti-human NOS2 antibody and 100 µg of protein extract per lane. NOS2 protein was not

found in the vector control cell lines (BaglacZ). VEGF protein concentrations were determined after immunoprecipitation of VEGF using 1 mg of protein extract. Molecular size (26-28 kDa) indicates the presence of the membrane-bound $VEGF_{189}$ splice form. Constitutive expression of VEGF in HCT-116 cells has been reported [34]. c, VEGF protein concentrations are higher (4.3 and 7.1-fold) in the culture medium of NOS2-expressing Calu-6 cell clones than it is in the culture medium of vector controls (A) and correlate with increased VEGF mRNA expression (B). The NOS-inhibitor L-NMA decreases VEGF secretion. $3x10^6$ cells were cultured in 4 ml of medium for 48 hr ± 2 mM L-NMA. VEGF was immunoprecipitated out of 1 ml of culture medium. The 4.4 kb VEGF mRNA was detected by Northern blotting with a 522 bp ^{32}P-labeled cDNA (Exon 1-7), and the 7.5 kb polycistronic mRNA encoding NOS2 [20] with the full-length human NOS2 cDNA [33].

Fig. 1a

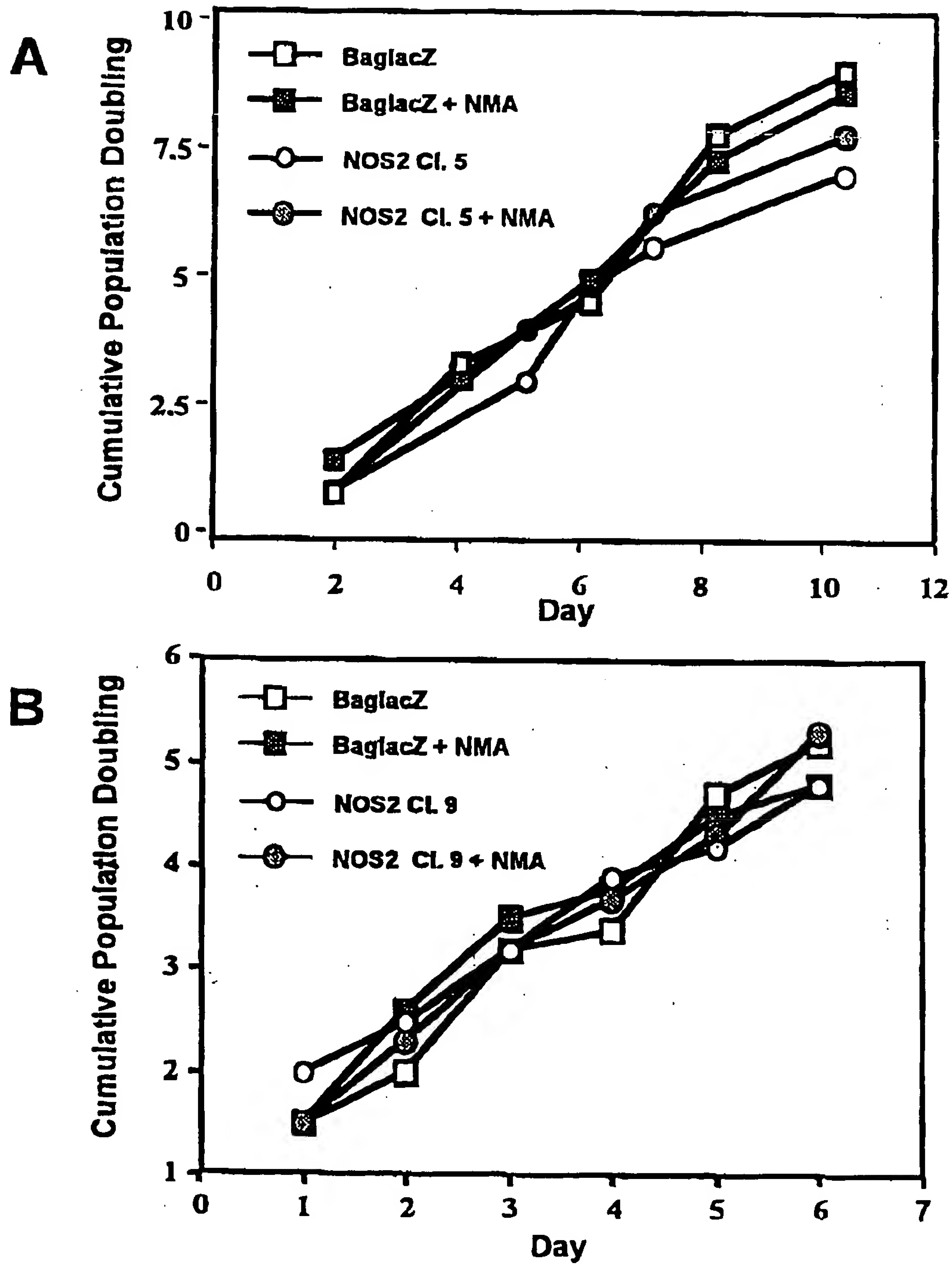
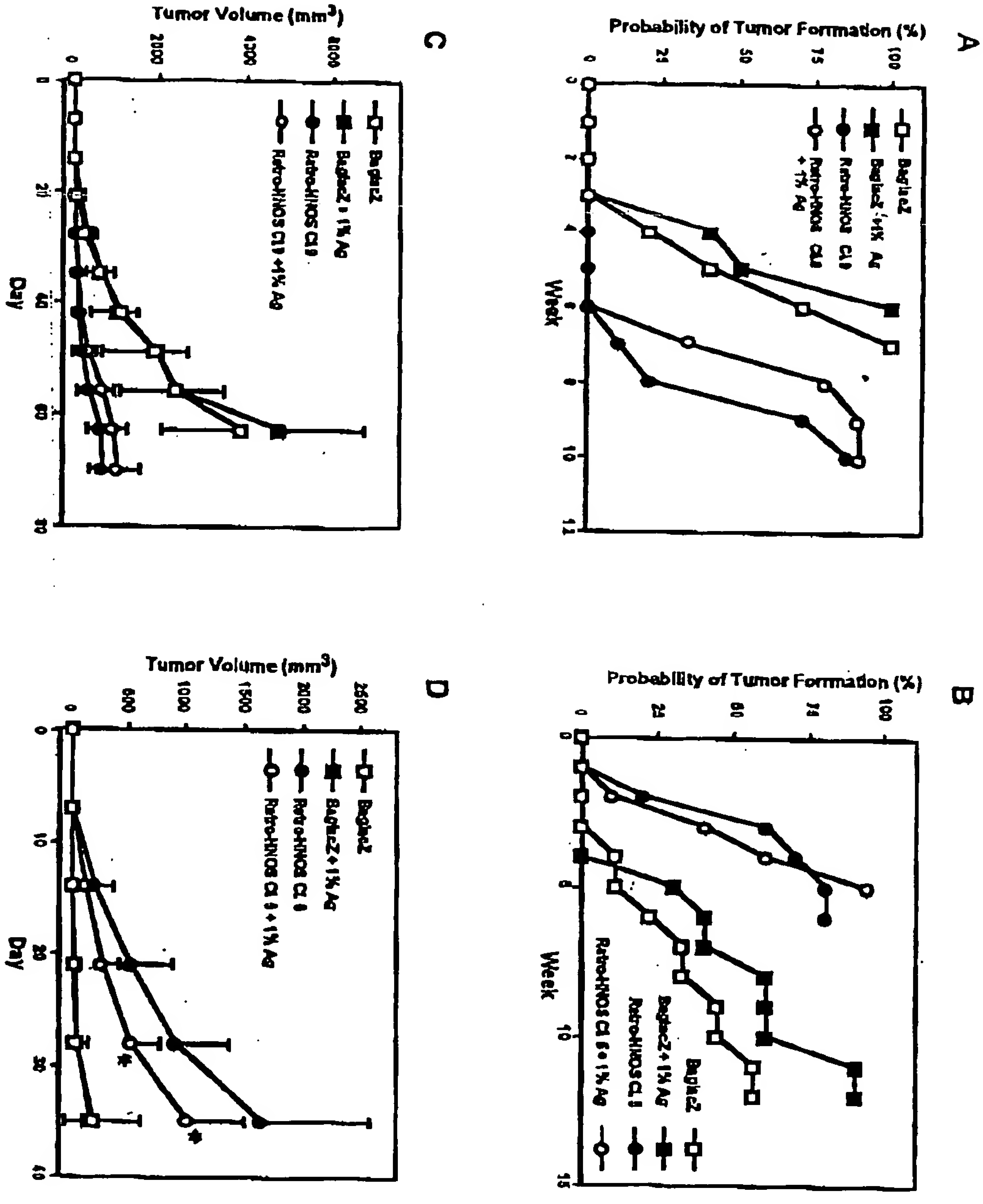
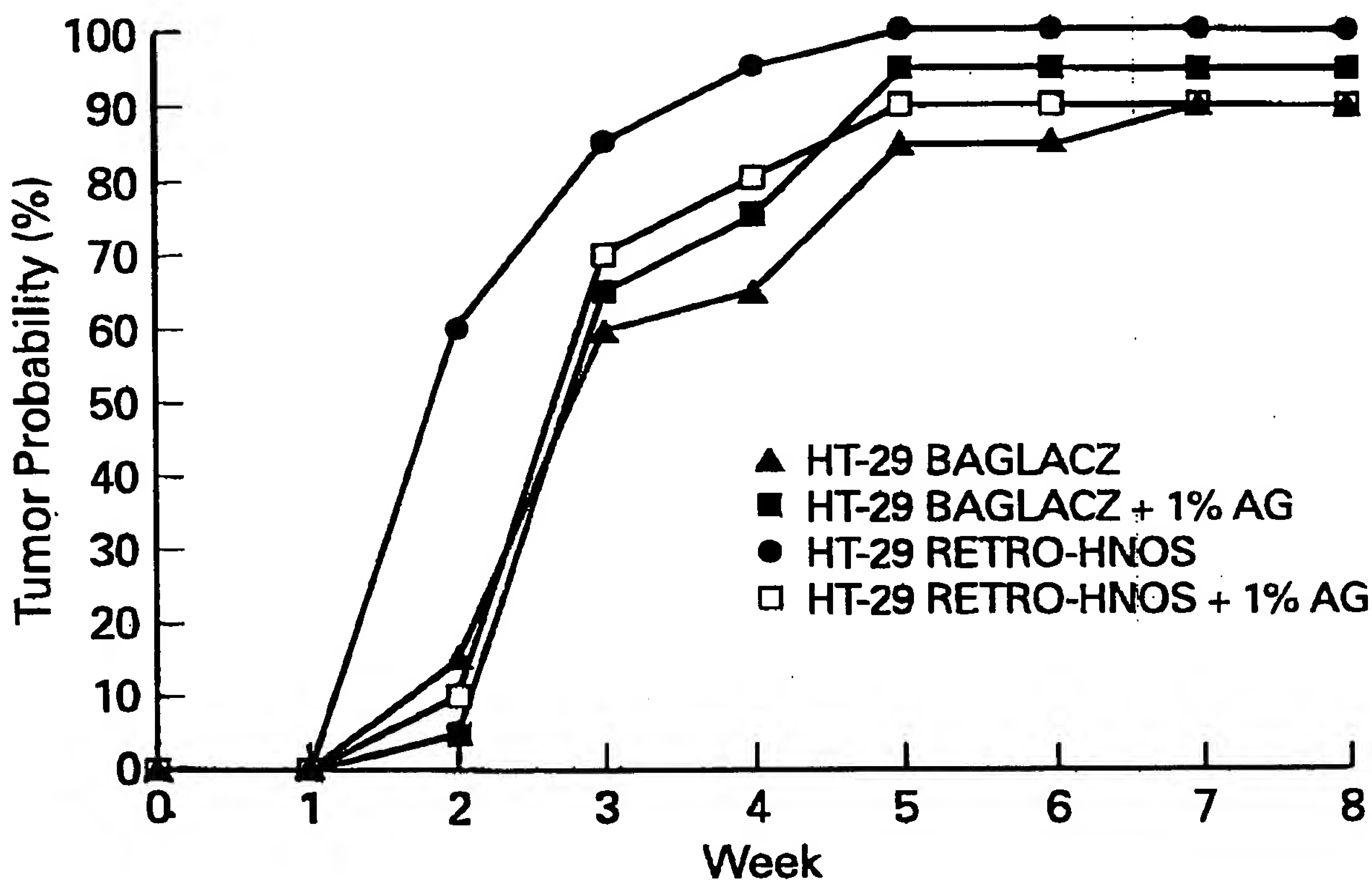

A
Cumulative Population Doubling
BaglacZ
BaglacZ + NMA
NOS2 Cl. 5
NOS2 Cl. 5 + NMA
Day
B
Cumulative Population Doubling
BaglacZ
BaglacZ + NMA
NOS2 Cl. 9
NOS2 Cl. 9 + NMA
Day

Fig 1b



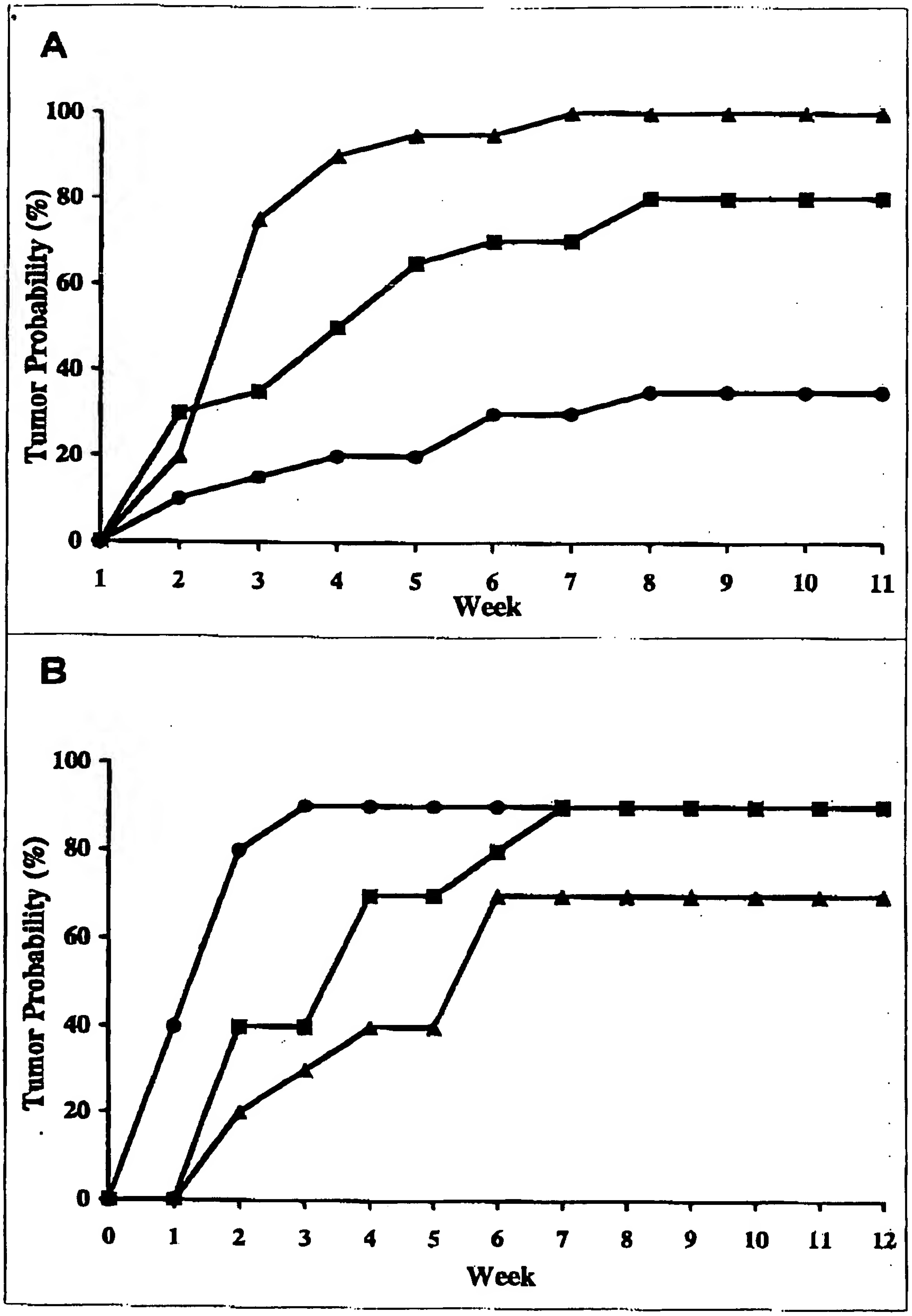
A
Probability of Tumor Formation (%)
Week
B
Probability of Tumor Formation (%)
Week
C
Tumor Volume (mm3)
Day
D
Tumor Volume (mm3)
Day

Fig. 1C



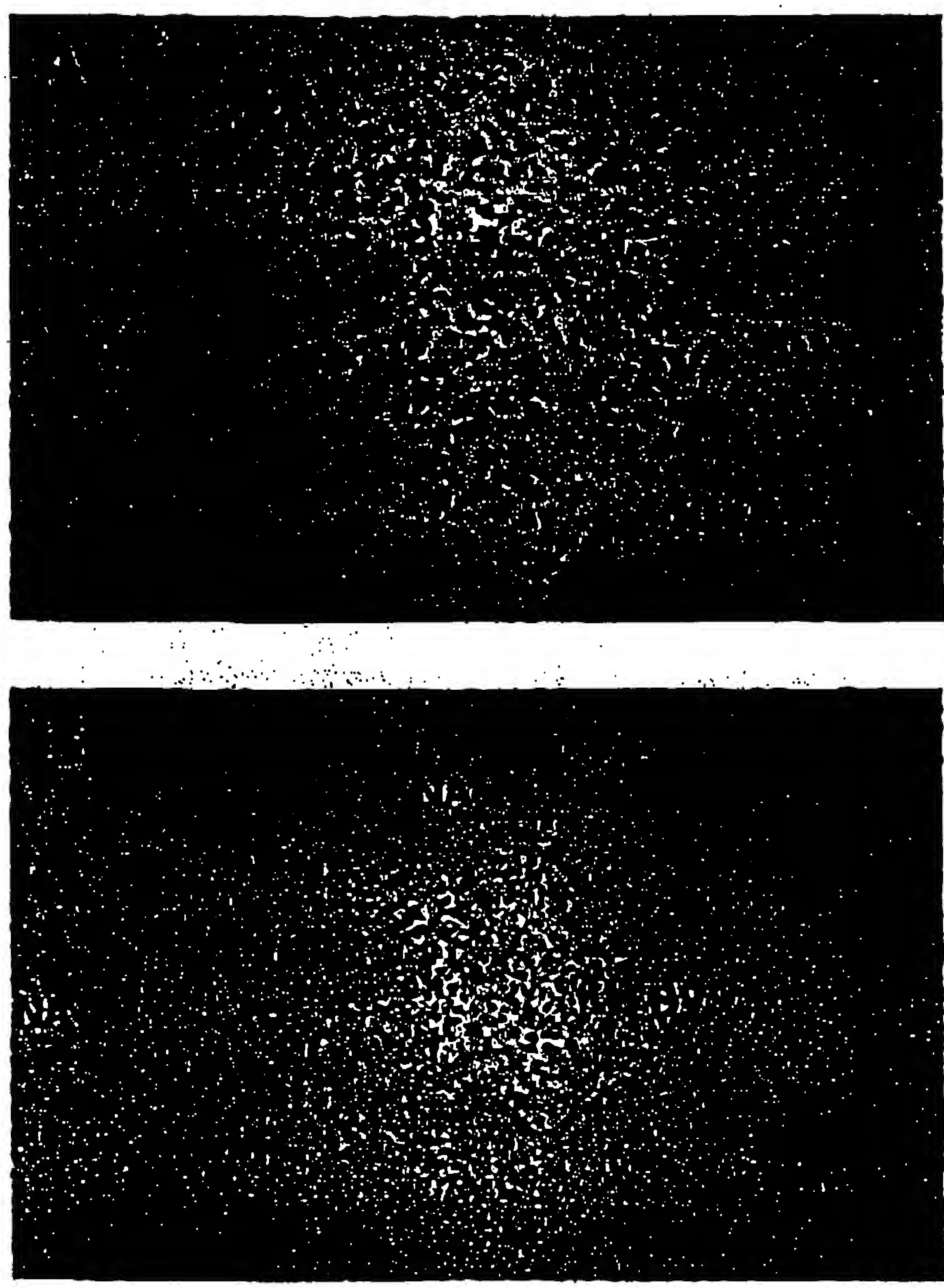
100
90
80
70
60
50
40
30
20
10
0
Tumor Probability (%)
0
1
2
3
4
5
6
7
8
Week
HT-29 BAGLACZ
HT-29 BAGLACZ + 1% AG
HT-29 RETRO-HNOS
HT-29 RETRO-HNOS + 1% AG

Fig. 1d



A
Tumor Probability (%)
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
Week
B
Tumor Probability (%)
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
10
11
12
Week

Fig. 2a



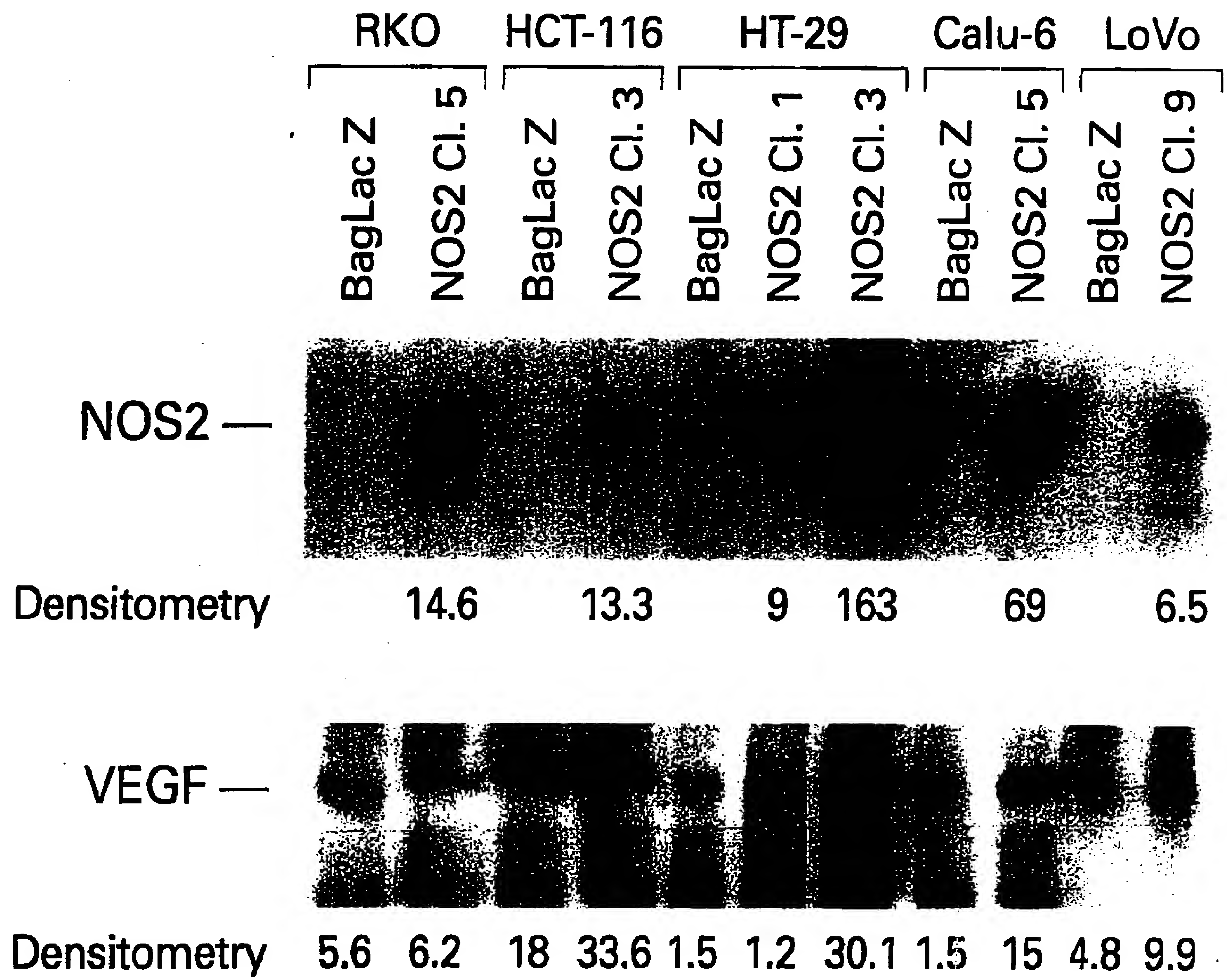
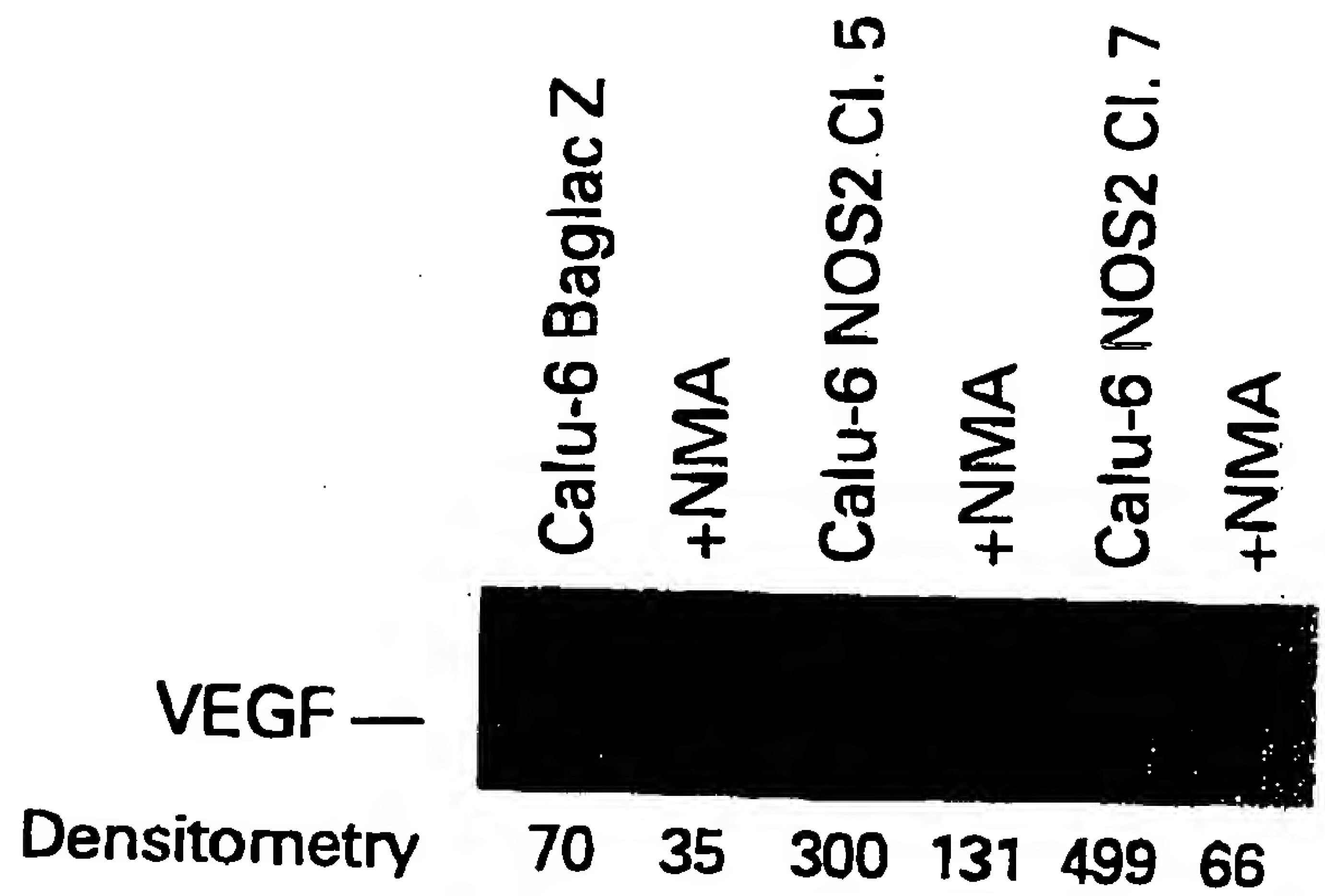

RKO
HCT-116
HT-29
Calu-6
LoVo
BagLac Z
NOS2 Cl. 5
BagLac Z
NOS2 Cl. 3
BagLac Z
NOS2 Cl. 1
NOS2 Cl. 3
BagLac Z
NOS2 Cl. 5
BagLac Z
NOS2 Cl. 9
NOS2
Densitometry 14.6 13.3 9 163 69 6.5
VEGF
Densitometry 5.6 6.2 18 33.6 1.5 1.2 30.1 1.5 15 4.8 9.9

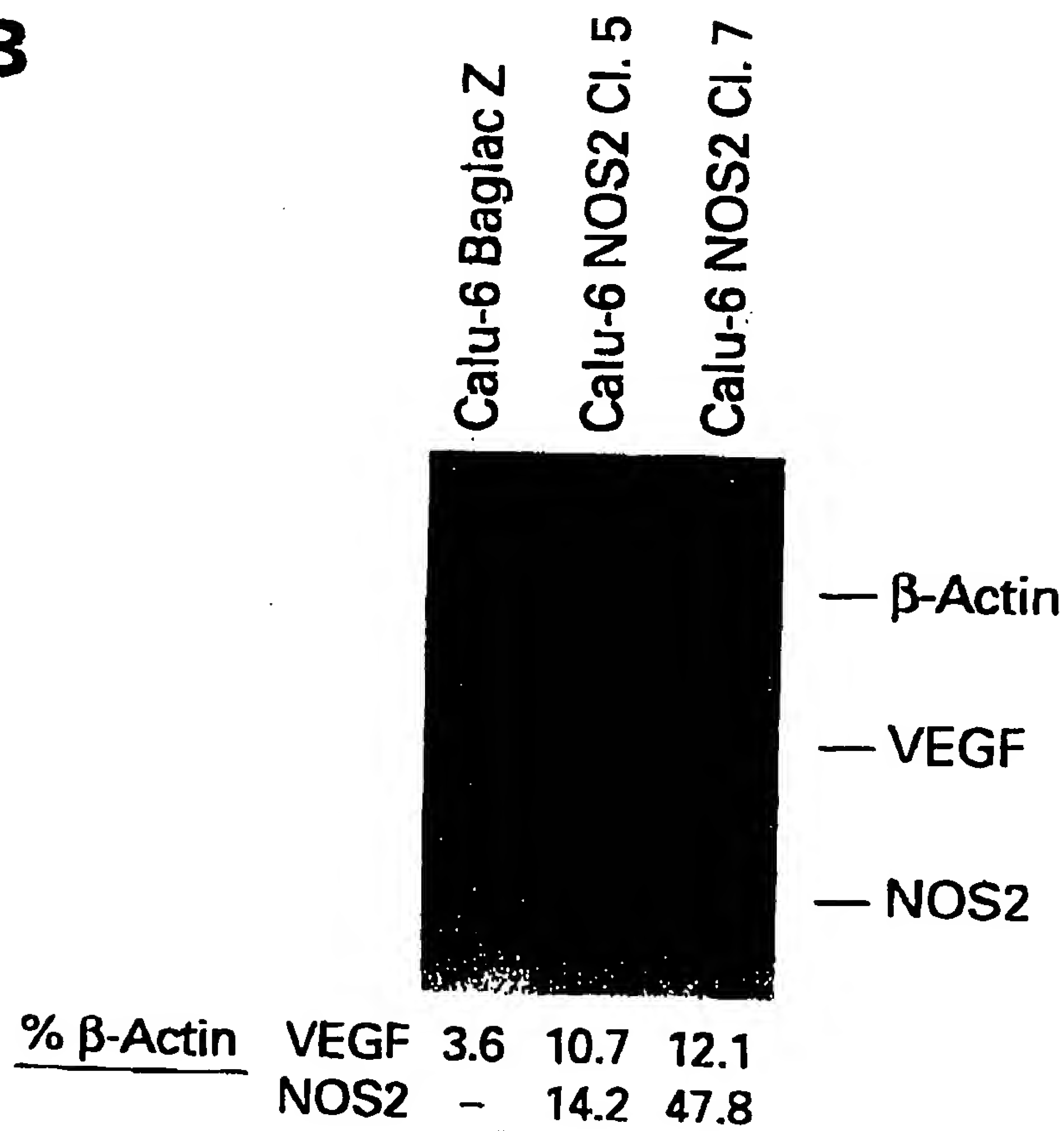
Fig. 26

Fig. 2c

A


Calu-6 Baglac Z
+NMA
Calu-6 NOS2 Cl. 5
+NMA
Calu-6 NOS2 Cl. 7
+NMA
VEGF
Densitometry 70 35 300 131 499 66

B


Calu-6 Baglac Z
Calu-6 NOS2 Cl. 5
Calu-6 NOS2 Cl. 7
β-Actin
VEGF
NOS2
% β-Actin VEGF 3.6 10.7 12.1
NOS2 – 14.2 47.8

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PHS Employee Invention Report

Use plain paper if more space is needed.

Part I: To Be Completed by the Inventor

First Inventor's Name: Curtis Harris Phone: 301/496-2048

1. Give a short descriptive title of your discovery or invention.

Cancer therapy using either nitric oxide producing drugs or inducible nitric oxide synthase inhibiting drugs depends on p53 status in the tumor.

2. Please provide (in non-scientific terms if possible) a one paragraph description of the essence of your discovery or invention and identify the public health need it fills.

The growth of xenotransplanted human cancer cells is dependent on nitric oxide-mediated induction of vascular endothelial growth factor and neovascularization. Importantly, the above discovery is valid only in human cancer cells containing a mutant p53 whereas cancer cells with wild-type (normal) p53 are growth inhibited. Because nitric oxide producing drugs are being considered as cancer chemotherapeutic agents, treatment of human cancers with mutant p53 could accelerate tumor growth and that inhibition of endogenous nitric oxide production by the inducible nitric oxide synthase (NOS-2) using NOS-2 inhibitor drugs would have therapeutic utility. Enclosed is a manuscript describing our invention in detail.

3. Who contributed to the invention or discovery? Please identify all colleagues who *could* merit co-authorship credit for the associated publication, whether or not you believe them to be "co-inventors."

Stefan Ambs and Curtis C. Harris generated the initial hypothesis and made the discovery. Timothy R. Billiar and David A. Geller provided the NOS-2 expression vector and William G. Merriam, Mofolusara O. Ogunfusika, William P. Bennett, Naoko Ishibe, Perwez S. Hussain, and Edith E. Tzeng contributed the technical and pathology aid. Whereas they warrant coauthorship, they did not generate the hypothesis to be tested or make the discovery.

4. Is anyone outside of the Public Health Service aware of your invention or discovery? If so, please identify them and describe the dates and circumstances.

Three of the coauthors, Timothy R. Billiar, Edith E. Tzeng and David A. Geller, are at the University of Pittsburgh.

5. Are you aware of any PHS patent applications that are related to your invention or discovery?

TDCB, NCI to check with Larry Keefer and David Wink at the NCI.

6. Please list the most pertinent previous articles, presentations or other public disclosures, made by you or by other researchers, that are related to your invention or discovery. Also, attach copies, ***please!***

None

7. Please indicate any future dates on which you will publish articles or make *any* presentations related to your invention or discovery.

The enclosed manuscript has been submitted and is under review by Nature Medicine.

8. In one paragraph, please speculate (and be creative!) about possible commercial uses of your invention or discovery.

Our discovery has at least two commercial implications. First, the use of cancer chemo-therapeutic agents based on either nitric oxide mediated-cytotoxicity or inhibition of NOS-2 activity will require determining the genetic and functional status of the p53 gene. Second, the search for the therapeutic activity of candidate nitric oxide producing drugs using cell- or xenograft-based screening assays will need to use cells with wild-type (normal) p53. The genetically engineered and characterized human tumor cell lines will be important reagents in a screening effort.

9. a. Is the subject matter of your invention related to a PHS CRADA (Cooperative Research and Development Agreement) involving your laboratory or ICD?
   - ☑ No
   - ☐ Yes. If yes, please identify the collaborator: ____________________

   b. Is the subject matter based on research materials that you obtained from some other laboratory?
   - ☐ No
   - ☑ Yes. If yes, please attach any material transfer agreements (MTA) under which you received the material.

10. What companies or academic research groups are conducting similar research (if you know)? Can you identify any companies that may be good licensing prospects?

Glaxo Wellcome, Searle, Amgen. Glaxo Wellcome has expressed an interest.

11. What further research would be necessary for commercialization of your invention? Generally, what are your future research plans for the invention and/or for research in areas related to the invention?

We plan to investigate the regulation of NOS-2 in human cancer cells and the mechanism by which nitric oxide inhibits the growth of human tumors containing wild-type (normal) p53 tumor suppressor gene.

12. Human Subject Certification: Does this invention rely upon data involving human subjects as defined in and regulated under 45 CFR Part 46?
    ☑ No ☐ Yes – If "yes," please provide the Institutional Review Board (IRB) protocol approval number and date: ____________________ *or* explain fully below:

13. First Inventor Information: *(Provide this information for each inventor who contributed to the essence of the invention. If more than one, use Page 4, "Information on Additional Inventors."*

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Affiliation
☒ ICD *(specify ICD and applicable box below)* LHC, DBS, NCI, NIH

☐ GS	☒ CO	☐ Visiting Scientist	☐ Special Volunteer
☐ GM	☐ Visiting Fellow	☐ Howard Hughes Fellow	☐ Other *(specify)*:
☐ SES	☐ Visiting Associate	☐ Guest Researcher	

☐ Non-ICD Affiliation *(specify)*:

If more than one inventor, what specific contribution did you make to this work?
Generated the hypothesis, directed the research and recognized the clinical importance of the discovery.

14. Inventors' Signatures

▶ This report is submitted pursuant to Executive Order 10096 and 10930 and/or Department Regulations. PHS employees have an obligation to report inventions they make while employed by PHS to OTT. Under E.O. 10096 and 367 CFR 501 the Government shall obtain the entire right, title, and interest in inventions: (i) made during working hours; or (ii) with Government facilities, equipment, materials, funds or information; or (iii) which bear a direct relationship or is made in consequence of the official duties of the inventor. If you are employed by PHS to conduct or perform research it is presumed that the invention was made under the foregoing circumstances. If this is not the case you must contact your Technology Development Coordinator (TDC) and provide the TDC with the details pertaining to this particular discovery or invention so that a determination of rights can be made.

Inventors' Signatures	Dates	Witnesses' Signatures	Dates
[signature]		Margaret M. Seddon	
Stefan Ambs		Anita Harrington	

Part II: To be completed by the Technology Development Coordinator.

15. Institute(s) or Agency(s) sponsoring this invention

National Cancer Institute, NIH

16. Patent prosecution fees are to be charged to

COPY

CAN:	To be provided at a later date.		
ICD:	NCI		

Authorizing Official *(Typed)*	Signature	Date
Karen Maurey	Karen Maurey	

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